



TITLE:

Extractives of *Quercus crispula* sapwood infected by the pathogenic fungi *Raffaelea quercivora* I: comparison of sapwood extractives from noninfected and infected samples

AUTHOR(S):

Imai, Kayoko; Mitsunaga, Tohru; Takemoto, Hiroyuki; Yamada, Toshihiro; Ito, Shin-ichiro; Ohashi, Hideo

---

CITATION:

Imai, Kayoko ...[et al]. Extractives of *Quercus crispula* sapwood infected by the pathogenic fungi *Raffaelea quercivora* I: comparison of sapwood extractives from noninfected and infected samples. *Journal of Wood Science* 2009, 55(2): 126-132

ISSUE DATE:

2009-04

URL:

<http://hdl.handle.net/2433/236168>

RIGHT:

The article, Extractives of *Quercus crispula* sapwood infected by the pathogenic fungi *Raffaelea quercivora* I: comparison of sapwood extractives from noninfected and infected samples, written by Kayoko Imai, Tohru Mitsunaga, Hiroyuki Takemoto, Toshihiro Yamada, Shin-ichiro Ito, Hideo Ohashi, was originally published electronically on the publisher's internet portal (currently SpringerLink) on December 27, 2008 without open access. With the author(s)' decision to opt for Open Choice the copyright of the article changed on April 27, 2018 to © The Author(s) 2018 and the article is forthwith distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits use, duplication, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) ...

## ORIGINAL ARTICLE

Kayoko Imai · Tohru Mitsunaga · Hiroyuki Takemoto  
Toshihiro Yamada · Shin-ichiro Ito · Hideo Ohashi**Extractives of *Quercus crispula* sapwood infected by the pathogenic fungi *Raffaelea quercivora* I: comparison of sapwood extractives from noninfected and infected samples**

Received: March 28, 2008 / Accepted: October 8, 2008 / Published online: December 27, 2008

**Abstract** The extracts of *Quercus crispula* infected by the ambrosia fungus, *Raffaelea quercivora*, were investigated. Phenol and tannin analyses indicated that normal sapwood (NS) contained a considerable amount of hydrolysable tannins, while infected colored sapwood (IS) contained less hydrolysable tannins and more phenols than NS. In treating pentagalloyl glucose (PGG), which is a model compound of hydrolysable tannins, with a culture medium of *R. quercivora*, PGG was rapidly hydrolyzed to produce gallic acid. The resulting gallic acid decreased in concentration over the subsequent cultivation period eventually disappeared. Measuring tannase and laccase activities of the culture medium of *R. quercivora*, tannase activity increased gradually from the beginning, while laccase activity increased rapidly at 5 days of incubation and disappeared at 8 days. An oxidative product from gallic acid treated with laccase was isolated by preparative high performance liquid chromatography, and was identified as purpogallincarboxylic acid (PGCA) by nuclear magnetic resonance spectroscopy and electron-impact mass spectrometry. PGCA was present in a 70% aqueous acetone extract of IS, and showed slight growth inhibition against *R. quercivora*.

**Key words** *Raffaelea quercivora* · *Quercus crispula* · Gal-  
lotannins · Ellagitannins · Tannase

**Introduction**

The mass mortality of oak trees has been widespread along the northwestern coastline of the Japanese island of Honshu since the late 1980s.<sup>1</sup> This large scale forest disease is caused by the ambrosia fungi, *Raffaelea quercivora*, which is a symbiotic microorganism with the ambrosia beetle, *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae).<sup>2</sup> The beetle carries the fungi in its mycangium, which is a storage organ used to carry symbiotic fungi for breeding its larva. The ambrosia beetle bores into the trunk of the dead or wilting oak tree and brownish-yellow coloration is generated along the beetle gallery in the sapwood. The colored sapwood may contain a number of substances, such as repellents against the beetle, because newly attacking beetles have an inclination to avoid colored parts of the trunk. However, knowledge of the chemical components and the biological function of the colored part are limited to date.

In some other cases, colorations in sapwood are formed by chemical reactions<sup>3,4</sup> or physiological reactions in living cells.<sup>5</sup> Kiln brown stain (KBS) that develops in kiln-dried radiata pine is a thermochemical reaction product of water-soluble compounds.<sup>4</sup> The presence of carbohydrates, cyclitols, amino acids, protein, and phenolics in the water-soluble compounds was reported and the reducing sugars and amino acids in the sap and hot-water extracts suggested that these compounds react together to form colored compounds.<sup>6</sup> This is the so-called Maillard-Amadori reaction,<sup>7,8</sup> which is commonly observed as food browning in cooking.

Colorations caused by enzymatic reactions have been reported for Douglas fir, *Pseudotsuga menziesii*,<sup>9,10</sup> and oak heartwoods.<sup>11</sup> Compounds existing in living cells of Douglas fir sapwood, such as *o*-dihydroxy phenols, (–)-epicatechin, and dihydroquercetin, react with naturally occurring

K. Imai · T. Mitsunaga (✉) · H. Ohashi  
Department of Applied Life Science, Faculty of Applied Biological  
Sciences, Gifu University, 1-1 Yanagido, Gifu 510-1193, Japan  
Tel. +81-58-293-2920; Fax +81-58-293-2920  
e-mail: mitunaga@gifu-u.ac.jp

H. Takemoto  
Center for Ecological Research, Kyoto University, Otsu, Shiga  
520-2113, Japan

T. Yamada  
Experimental Section at Chiba, The University Forest, The  
University of Tokyo, Kamogawa, Chiba 299-5503, Japan

S. Ito  
Department of Sustainable Resource Sciences, Faculty of  
Bioresources, Mie University, Tsu, Mie 514-8507, Japan

Part of this study was presented at the 57th Annual Meeting of the  
Japan Wood Research Society, Hiroshima, Japan, 2007

enzymes in the trees and form brown-colored materials in the exposed surface of the lumber. Bauch et al.<sup>11</sup> found that the yellow coloration in *Quercus robur* L. and *Quercus petraea* is caused by a mold fungus, *Paecilomyces variotii* Bain., and that the reacting compounds are hydrolysable tannins. The tannase of *Paecilomyces variotii* Bain.<sup>12</sup> would release the reactants that would then form the coloration.

This study clarifies the relationship between the extracts of *Quercus crispula* sapwood and crude enzyme of *R. quercivora* to understand the role of the extracts in the colored sapwood of *Q. crispula*.

## Materials and methods

### Analytical instruments

High performance liquid chromatography (HPLC) used a reversed-phase column (Inertsil ODS-3V, 4.6 mm i.d. × 250 mm, GL Science, Japan) and a diode array detector (SPD-M10A vp, Shimadzu). The following solvent system was used: a linear gradient elution for 45 min from 5% to 100% methanol in 0.05% trifluoroacetic acid (TFA) in H<sub>2</sub>O at a flow rate of 1 ml/min, monitored at 280 nm.

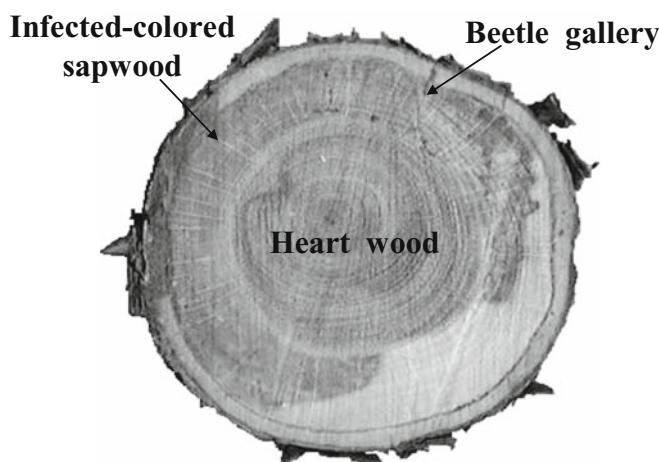
Preparative HPLC was performed on a Develosil packed column (packing Lop ODS size 24S) with elution at 10 ml/min under isocratic condition (50 parts of acetonitrile containing 0.1 % TFA and 50 parts of 0.1 % TFA).

Ultraviolet (UV) spectra were measured on a Jasco V-520 spectrophotometer and electron-impact mass spectrometry (EI-MS) was performed on a Shimadzu GCMS-QP5050 instrument. <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL ECA-500 NMR spectrometer (500 MHz), and chemical shifts are given in δ (ppm) values relative to that of the solvent [methanol-*d*<sub>4</sub> (δ<sub>H</sub> 3.35; δ<sub>C</sub> 49.0)].

### Extraction of *Quercus crispula* sapwood

Normal sapwood of *Quercus crispula* was collected in the Kuraiyama experimental forest, Gifu University, in November 2006. This wood was separated into bark, sapwood, and heartwood. The sapwood was milled into a powder (580.6 g) (ZM200 Ultra Centrifugal Mill, Retsch), which was then soaked in 70% aqueous acetone (35.9 l). After evaporating the acetone, the solution was extracted successively with diethyl ether (Et<sub>2</sub>O) (3 l × 3), ethyl acetate (EtOAc) (3 l × 8), and *n*-butanol (*n*-BuOH) (3 l × 4) to give the respective extracts. All extracts were analyzed by HPLC. The EtOAc-soluble fraction (EtOAc-S) was used as a sample in subsequent experiments.

Infected colored sapwood of *Q. crispula* (Fig. 1) was collected at the Toyama Forest and Forestry Products Research Center in November 2007. The wood (infected colored sapwood) was separated and ground and the powder (725.6 g) was extracted with 70% aqueous acetone (11.0 l). An aliquot of the extract was analyzed by HPLC.



**Fig. 1.** Cross section of *Quercus crispula* attacked by *Platypus quercivorus*

No large difference was found when we analyzed extracts of *Q. crispula* sapwood obtained earlier from Toyama, Kyoto, Ishikawa, and Gifu (data not shown). As a result, noninfected and infected *Q. crispula* was used in this study regardless of its original location.

### Chemical analyses of extracts

Tannin analyses were conducted as reported in previous studies.<sup>13,14</sup> Total phenol and flavanol contents were measured by the Folin-Ciocalteu method and the vanillin-hydrochloric acid method, respectively, using (+)-catechin as a standard reference.

Total phenol and total flavanol were calculated as follows:

$$\text{Total phenol or total flavanol (\%)} = 100 \times Y/S,$$

where *Y* represents the concentration of (+)-catechin (mg/ml) calculated from a calibration curve and *S* represents concentration (mg/ml) of the sample [normal sapwood (NS) or infected sapwood (IS) extracts].

The adsorption of bovine serum albumin (BSA) by the extracts was evaluated by measuring the amount of BSA remaining in the supernatant after centrifugation of the reacting mixture; the residue contained precipitates formed by reaction of extract components with BSA. NS or IS extract (1 mg/ml in 50% ethanol) and BSA aqueous solution (5 mg/ml) were mixed in equal volumes and kept at room temperature for 1 h. Then BSA concentration was measured by HPLC analysis. BSA adsorption was calculated as follows:

$$\text{BSA adsorption (\%)} = (A - B)/A \times 100,$$

where *A* is the BSA concentration in the control solution, and *B* is the BSA concentration in the solution after mixing with samples (NS or IS extracts). The concentration of BSA was calculated from a calibration curve.

Hydrolysis was conducted as follows. Five milligrams of NS extract was treated with 5 ml of 3 N sulfuric acid at 110°C for 10 h. After the temperature was lowered to room temperature, 6 N sodium hydroxide was added. The hydrolysate was analyzed by HPLC.

Enzymatic reaction of pentagalloyl glucose treated with crude enzyme of *Raffaelea quercivora*

Pentagalloyl glucose (PGG) was isolated from tannic acid by preparative HPLC. Isolated PGG was analyzed by NMR spectroscopy.<sup>15</sup>

For cultivation of *Raffaelea quercivora*, liquid medium including vitamins and minerals<sup>16</sup> was used. Ten milliliters of liquid medium was sterilized by autoclave (121°C, 15 min). After sterilization, 20 µl of PGG solution (200 mg/ml, soluble in 50% methanol) was added and *R. quercivora* was inoculated. After incubation for 1, 2, 4, 6, 9, and 11 h and every day up to 12 days, an aliquot of the medium was analyzed by HPLC. The amounts of PGG and gallic acid in the medium were calculated from calibration curves derived from HPLC analysis of authentic PGG and gallic acid.

Hydrolysable tannins of *Quercus crispula* treated by crude enzyme of *Raffaelea quercivora* or commercial tannase

*Raffaelea quercivora* was inoculated into 10 ml of sterilized culture medium including 20 µl of EtOAc-S solution (20 mg/100 µl in 50% MeOH). After 10 days of incubation at 28°C, an aliquot of culture medium was centrifuged (12 500 rpm, 5 min). Then 600 µl of supernatant was extracted with 400 µl of EtOAc; 200 µl of the EtOAc-soluble fraction was dried and analyzed by HPLC.

On the other hand, EtOAc-S was treated with commercial tannase derived from *Aspergillus oryzae* (Wako, Japan) as follows. EtOAc-S was dissolved in 0.05 mol/l citric acid buffer (pH 5.5) to give a concentration of 1.6 mg/450 µl. One thousand microliters of tannase solution (3 mg/10 ml in citric buffer) was added to 4000 µl of EtOAc-S solution and kept at 30°C, then 200 µl of EtOAc was added to stop the reaction. Finally, an aliquot of the EtOAc-soluble fraction was collected and analyzed by HPLC.

## Enzyme activities

Culture medium including minerals and vitamins<sup>16</sup> (10 ml) was sterilized and inoculated with precultured *R. quercivora* [on potato dextrose agar (PDA) medium]. The culture medium was incubated for 10 days, during which an aliquot of the culture medium was periodically collected and filtered and the enzyme activity of the filtrate measured.

For the measurement of tannase activity, crude enzyme solution preincubated at 35°C for 10 min and 100 µl of 10 mg/ml tannic acid solution in 0.05 M citrate buffer (pH

5.5) were mixed and incubated at 35°C for 1 h; 1 N hydrochloric acid was added to stop the enzymatic reaction. The concentration of gallic acid in the reaction mixture was measured by HPLC analysis.

Laccase activity was measured using syringaldazine as substrate. Two hundred microliters of crude enzyme extract, 1.2 ml of 0.1 M acetate buffer (pH 5.7), and 200 µl of 0.05 mM syringaldazine ethanol solution were mixed. The rate of oxidation of syringaldazine to quinone was monitored at 525 nm.

For peroxidase activity, 1 M guaiacol solution, 0.1 M phosphate buffer (pH 7.4), 30 mM hydrogen peroxide solution, and crude enzyme solution were kept at 20°C before the activity test. One milliliter of 1 M guaiacol solution, 1 ml of 0.1 M phosphate buffer, and 1 ml of crude enzyme solution were mixed and 0.027 ml of 30 mM hydrogen peroxide was added to the reaction solution to start the reaction. Oxidation of guaiacol was measured by the increase in the absorbance at 470 nm.

## Identification

Five hundred milliliters of culture medium including minerals and vitamins<sup>16</sup> was sterilized and precultured *Pycnoporus coccineus*, which is a known white rot fungus that produces a laccase, on PDA medium was inoculated and cultured at room temperature with shaking. After 5 days of incubation, the fungus body was removed by filtration and the resulting filtrate was dialyzed against buffer solution with Spertra/Por (molecular weight cutoff 12 000–14 000) for 24 h.

In order to identify the product of the enzymatic reaction, 200 mg of gallic acid dissolved in 0.05 M sodium acetate buffer (pH 5.0) was added to 5 ml of extracellular crude enzyme from *Pycnoporus coccineus* and incubated at 37°C for 1 week. The reaction mixture was evaporated and the residue was applied to a Sephadex LH-20 column (26 mm i.d. × 200 mm) using water as eluent to separate the yellow-colored fraction, mainly containing the product. The main compound, purprogallincarboxylic acid (PGCA), was isolated as a brownish crystal by preparative HPLC with an ODS column, and was identified by NMR spectroscopy and EI-MS.

*Purprogallincarboxylic acid*. UV (MeOH) λ<sub>max</sub>: 224, 304, 398 nm. EI-MS (70 eV) *m/z*: 264 [M]<sup>+</sup>, C<sub>12</sub>H<sub>8</sub>O<sub>7</sub>. <sup>1</sup>H NMR (dimethyl sulfoxide-*d*<sub>6</sub>, 500 MHz): δ 7.14 (1H, H-1), 7.77 (1H, H-7), 8.22 (1H, H-9). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 125 MHz): δ 112.71 (C-1), 114.81 (C-9a), 115.81 (C-7), 130.98 (C-4a), 136.61 (C-8), 136.66 (C-9), 136.70 (C-3), 151.89 (C-4), 152.54 (C-2), 152.64 (C-6), 168.45 (C-α), 181.98 (C-5). HPLC analysis was performed to confirm the presence of PGCA in IS. The chromatogram was measured at 400 nm (λ<sub>max</sub> of PGCA). Conditions: reversed-phase column (Develosil HG-5 4.6 mm i.d. × 250 mm); linear gradient elution for 45 min from 5% to 90% methanol in 0.01% trifluoroacetic acid solution; flow rate 1 ml/min.



## Antifungal assays

Forty milliliters of sterilized PDA medium was poured into a 15-cm-diameter petri dish. An ethanol solution of gallic acid, ellagic acid, or PGCA (5 mg/ml) was spread on PDA medium. After the ethanol was evaporated completely, *R. quercivora* was placed on the center of the petri dish and the dish was incubated at 28°C for 3 days. The experiments were performed in triplicate and the diameter of *R. quercivora* was measured every day.

## Statistical analysis

Significant difference between control and samples (gallic acid, ellagic acid, and PGCA) was assessed by one-way analysis of variance and  $P < 0.05$  was considered as significant.

## Results and discussion

Total phenol, total flavanol values, and BSA adsorption of extracts of *Quercus crispula* sapwood

Some chemical analyses were conducted on the extracts from normal sapwood (NS) and infected colored sapwood (IS) (Table 1). Total phenol content of NS and IS extracts were 47.3% and 85.6%, and their BSA precipitations were 59.3% and 71.6%, respectively. On the other hand, total flavanol content of both extracts were less than 1% based on the extracts. Judging from the result, the extracts of *Q. crispula* showed little or no flavanol content, so the extracts would not contain condensed tannins. Therefore, these results indicate that NS and IS extracts contain hydrolysable tannin-like substances (not proanthocyanidin type).

## Comparison of NS and IS extracts by HPLC

According to HPLC analysis (Fig. 2) with a photodiode array monitor, there were several phenolic compounds with  $\lambda_{\max}$  values above 300 nm in the NS and IS extracts. However, chromatographically there was a difference between these two extracts; in the NS chromatogram, broad peaks appeared at a retention time of about 30 min. On the other hand, the HPLC chromatogram of the hydrolysate of NS, hydrolyzed with 3 N sulfuric acid, shows that there are several peaks related to phenolics, and ellagic acid a small amount of gallic acid are also present. Therefore, hydro-

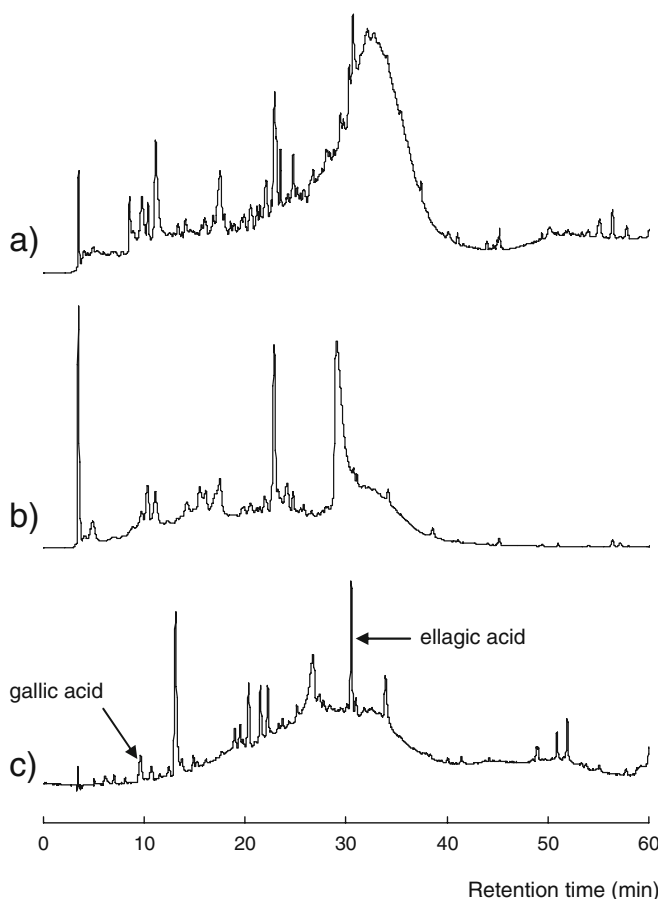
lysable tannins, especially ellagitannins, exist in the NS extracts.

## Quantity of PGG and gallic acid in the culture medium of *Raffaelea quercivora*

Hydrolysable tannins are classified roughly into gallotannins and ellagitannins. PGG is a representative gallotannin and a constituent of commercial tannic acid.

After PGG was added to the culture medium (4  $\mu$ g/10 ml culture medium) of *Raffaelea quercivora*, the amounts of PGG and gallic acid were measured over time by HPLC and are shown in Fig. 3. The amount of PGG fell to half in 11 h and completely disappeared in 2 days. On the contrary, the amount of gallic acid increased gradually, suggesting that *R. quercivora* produces hydrolytic enzyme, such as tannase, in the culture medium.

On the other hand, the amount of gallic acid was increased with the decrease of PGG, reached a maximum at 5 days, and disappeared after 11 days of incubation. Theoretically, 5 moles of gallic acid is produced from 1 mole of PGG by hydrolysis. In this experiment, 4 mg (4.26  $\mu$ mol, 0 h) of PGG was hydrolyzed to produce only 9.08  $\mu$ mol of



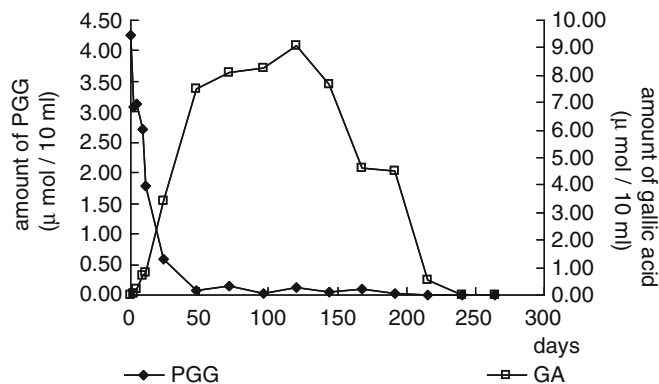
**Fig. 2a-c.** High performance liquid chromatography (HPLC) chromatograms of extracts from *Q. crispula* sapwood with detection at 280 nm. **a** Extracts from normal sapwood of *Q. crispula* (NS); **b** extracts from infected colored sapwood of *Q. crispula* (IS); **c** NS hydrolyzed with 3 N  $H_2SO_4$  at 110°C for 10 h

**Table 1.** Chemical analysis of the extracts of *Quercus crispula*

Sample	Total phenol (%)	Total flavanol (%)	Protein precipitation (%) <sup>a</sup>
NS	47.3	0.18	59.3
IS	85.6	0.25	71.6

Data expressed as percentage based on dry NS and IS  
NS, Normal sapwood; IS, infected colored sapwood

<sup>a</sup> Calculated as described in text



**Fig. 3.** Amount of pentagalloyl glucose (PGG) and gallic acid (GA) in the culture medium of *R. quercivora*

gallic acid as a maximum (120 h). Therefore, production and consumption of gallic acid must have taken place at the same time in the culture medium.

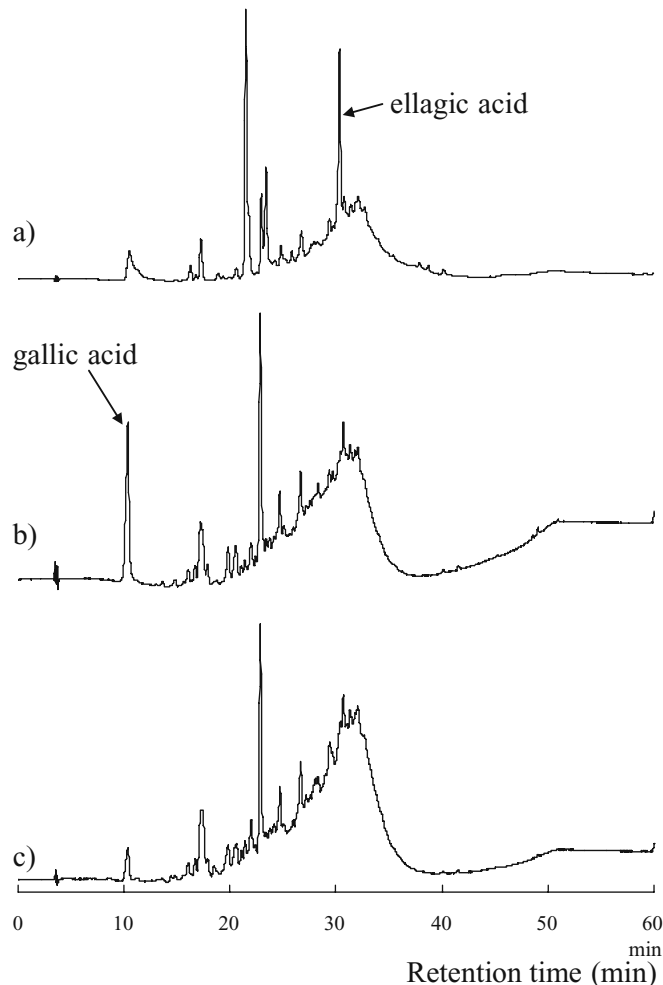
#### Characteristic property of tannase-like enzyme from *Raffaelea quercivora*

Crude enzyme solution from *R. quercivora* showed tannase-like activity for PGG. In order to confirm the activity of the crude enzyme solution against the extracts of *Q. crispula*, EtOAc-S was treated with the crude enzyme solution.

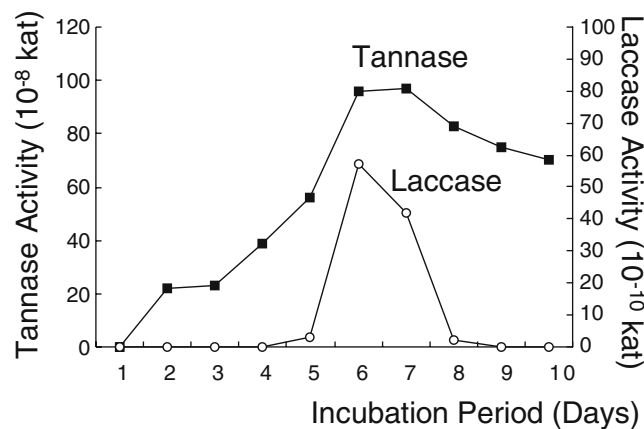
Figure 4 shows the HPLC chromatograms of the EtOAc-S solution treated with the crude enzyme of *R. quercivora* or commercial tannase derived from *Aspergillus oryzae*. In treating EtOAc-S with the crude enzyme, a small amount of gallic acid ( $R_f$  10 min) and a large amount of ellagic acid ( $R_f$  31 min) were produced with a decreasing series of peaks at around 30 min retention time. On the other hand, a remarkable amount of gallic acid and little or no ellagic acid were produced with a slight decrease of the series of peaks in case of using commercial tannase. It is possible that a tannase of *R. quercivora* may have a unique ability to hydrolyze both gallotannins and ellagitannins.

#### Crude enzyme activity of *Raffaelea quercivora*

In a manner that is similar to the hydrolysis of PGG as shown in Fig. 3, the amount of gallic acid produced from EtOAc-S and the crude enzyme of *R. quercivora* increased up to a specific incubation time, after which it decreased gradually and finally disappeared (data not shown). Such a decrease in gallic acid could be due to the presence of phenol oxidase in the crude enzyme solution. Figure 5 shows the tannase and laccase activities of the crude enzyme solution for 10 days. The tannase activity of *R. quercivora* gradually increased from the beginning of the incubation and peaked on the sixth day of incubation. Peroxidase activity was not detected. The crude enzyme solution has laccase activity which shows a drastic increase on the sixth day of incubation. It is indicated that *R. quercivora* has the ability to produce tannase and laccase.



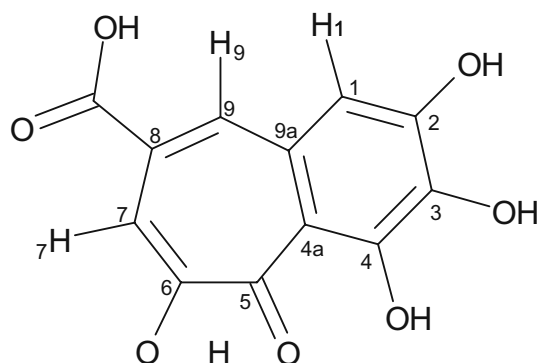
**Fig. 4a-c.** HPLC chromatograms of the ethyl acetate-soluble extract of normal sapwood (EtOAc-S) treated with **a** crude enzyme of *R. quercivora*, **b** commercial tannase. **c** Blank



**Fig. 5.** Tannase and laccase activities in crude enzyme solution of *R. quercivora* incubated at 35°C

#### Identification of purprogallincarboxylic acid

The enzymatic oxidation of gallic acid with the laccase was performed for 7 days at room temperature. Addition of laccase solution to gallic acid solution gradually darkened



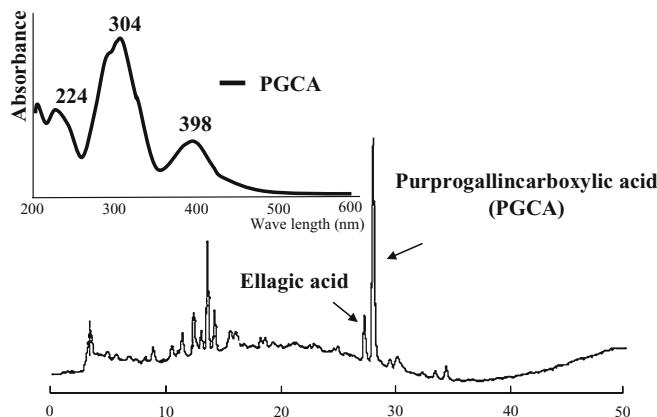
**Fig. 6.** Structure of purrogallincarboxylic acid (PGCA)

the color of the reaction mixture. One compound with  $\lambda_{\text{max}}$  values of 224, 304, and 398 nm was found by HPLC analysis of the colored reaction mixture. The  $^1\text{H}$  NMR spectrum showed three singlets at 7.14, 7.77, and 8.22 ppm and no other proton resonance was observed. Twelve carbon signals including a carboxyl group at 160 ppm and a conjugated carbonyl group at 182 ppm were observed in the  $^{13}\text{C}$  NMR spectrum. According to the C-H COSY two-dimensional (2D) correlation spectrum and the DEPT NMR spectrum, the three protons are directly attached to each corresponding methine carbon (H-C correlations:  $^1\text{H}$  7.14 ppm and  $^{13}\text{C}$  112.71 ppm,  $^1\text{H}$  7.77 ppm and  $^{13}\text{C}$  115.81 ppm, and  $^1\text{H}$  8.22 ppm and  $^{13}\text{C}$  136.66 ppm). By understanding C-H correlations with heteronuclear multi-bond correlation (HMBC) 2D NMR spectroscopy, some partial structures of the compound were clarified. Although it was impossible to observe the molecular ion peak by EI-MS, all information indicated the compound was purrogallincarboxylic acid (Fig. 6), which was identified and reported as an oxidative product from gallic acid converted with a polyphenol oxidase from potato.<sup>17</sup>

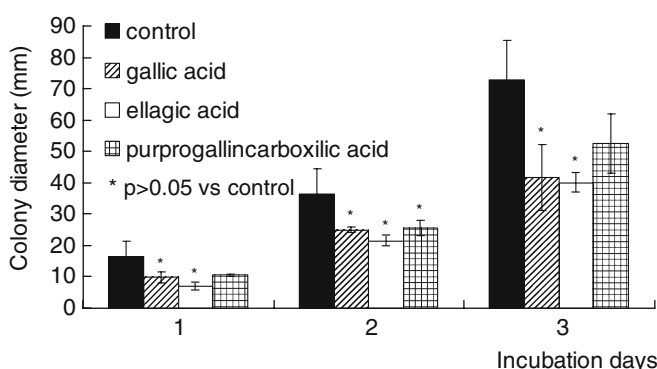
According to the HPLC analysis with detection at 400 nm, the water-soluble fraction of IS extracts showed a well-defined peak at a retention time of 28 min (Compound 1) as shown in Fig. 7. The UV spectrum and retention time in HPLC of Compound 1 conformed with those of PGCA. This suggests that PGCA is present in infected colored sapwood of *Q. crispula*, and indicates that PGCA is a significant contributor to the coloration caused by *R. quercivora* in diseased sapwood of *Q. crispula*.

#### Antifungal activities of gallic acid, ellagic acid, and PGCA

Figure 8 shows the antifungal activities of gallic acid, ellagic acid, and PGCA against *R. quercivora*. After 1 day of incubation, the mycelium diameters were almost the same, but after 3 days of incubation, gallic acid inhibited the mycelium growth by about 43%, ellagic acid by about 45%, and PGCA by about 28%. This suggests that gallic acid and ellagic acid have antimicrobial activities against *R. quercivora* (significantly different from control;  $P < 0.05$ ). Compared with the antimicrobial activities of gallic acid and



**Fig. 7.** HPLC chromatogram with detection at 400 nm of the water-soluble fraction of IS extracts. The ultraviolet absorption spectrum and the HPLC retention time of Compound 1 conformed with those of PGCA



**Fig. 8.** Antifungal activities of gallic acid, ellagic acid, and PGCA against *R. quercivora*

ellagic acid, that of PGCA was lower and was not significantly different from the control.

Gallic acid has antimicrobial activity against some microorganisms, including *Staphylococcus aureus*,<sup>18</sup> *Escherichia coli*,<sup>18</sup> and *Fusarium fusiformis*.<sup>19</sup> It was thought that gallic acid might also be toxic against *R. quercivora*; thus, it appears that *R. quercivora* is equipped with the means to change the toxic compound (gallic acid) into the low toxic compound (PGCA).

In conclusion, *R. quercivora* hydrolyzes hydrolysable tannins to produce gallic and ellagic acids in sapwood and prevents the formation of complex products from reaction between enzymes and tannins. However, the resulting polyphenols are not favorable for growth of *R. quercivora*, so the fungus converts the polyphenols into nontoxic compounds by enzymatic oxidation, which causes coloration of the sapwood.

**Acknowledgments** The authors are grateful to Dr. Koji Takabata (Toyama Forestry and Forest Products Research Center) for providing the infected *Q. crispula* plants. This research was partially supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant-in-Aid for Scientific Research (B), 16380096.

## References

1. Ito S, Kubono T, Sahashi N, Yamada T (1998) Associated fungi with the mass mortality of oak trees (in Japanese). *J Jpn For Soc* 80:170–175
2. Kubono T, Ito S (2002) *Raffaelea quercivora* sp. nov. associated with mortality of Japanese oak and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* 43:255–260
3. Bauch J (1986) Verfärbungen von Rund- und Schnittholz und Möglichkeiten für vorbeugende Schutzmaßnahmen. *Holz-Zentralbl* 112:2217–2218
4. Armondo GM, Marc F, Bernhard K (1997) Chemical and UV-VIS spectroscopic study on kiln brown stain formation in radiata pine. In: *Proceedings of the 9th International Symposium of Wood and Pulp Chemistry*. Montreal, Canada, June 9–12, pp 70.1–70.5
5. Lidija M (1975) Formation of tyloses in felled *Quercus rubra* L. *Wood Sci Technol* 9:3–14
6. Armondo GM, Marc F, Bernhard K, Frank L (2000) The chemical nature of kiln brown stain in radiata pine. *Holzforschung* 54: 12–22
7. Rizzi GP (1994) The Maillard reactions in food. In: Labuza TP, Reineccius GA, Morrier VM, O'Brien J, Baynes JW (eds) *Maillard reactions in chemistry, food and health*. Royal Society of Chemistry, Cambridge, UK, pp 11–19
8. Threander O, Nelson DA (1988) Aqueous high temperature transformation of carbohydrates relative to utilisation of biomass. *Adv Carbohydr Chem Biochem* 46:273–326
9. Murray LL, Durria AAM (1996) An enzyme extract from Douglas-fir sapwood and its relationship to brown staining. *Wood Fiber Sci* 28:2–6
10. Laver ML, Durria AAM (1997) Chemical brown staining of Douglas-fir wood: characterization of a wood enzyme extract. *Forest Prod J* 47:93–97
11. Bauch J, Hundt H, Weissmann G, Lange W, Kubel H (1991) On the causes of yellow discolourations of oak heartwood (*Quercus* sect. *robur*) during drying. *Holzforschung* 45:79–85
12. Battestin V, Macedo GA (2006) Tannase production by *Paecilomyces variotii*. *Bioresource Technol* 98:1832–1837
13. Takagi K, Shimomura K, Koizumi Y, Mitsunaga T, Abe I (1999) Tyrosinase inhibitors from the pericarp of jatoba (*Hymenaea courbaril* L.) (in Japanese with English summary). *Nat Med* 53:15–21
14. Takagi K, Mitsunaga T (2002) A tyrosinase inhibitor from the asam (*Mangifera quadrifida*). *Nat Med* 56:97–103
15. Richard T, Vitrac X, Merillon JM, Monti JP (2005) Role of peptide primary sequence in polyphenol-protein recognition: an example with neurotensin. *Biochim Biophys Acta* 1726:238–243
16. Yoshihara I, Akamatsu I, Ueshima H, Fujii T (1985) Screening of white rot fungi which has strong resolution activity against lignin (in Japanese). *Jpn TAPPI J* 39:65–74
17. Karl H (1954) Über die Bildung der Purpurogallincarbonsäure durch fermentative Oxydation der Gallussäure. (zugleich II Mitteilung über gerbstoffartige Substanzen). *Archiv der Pharmazie* 287:497–503
18. Panizzi L, Caponi C, Catalano S, Cioni PL, Morelli I (2002) In vitro antimicrobial activity of extracts and isolation constituents of *Rubus ulmifolius*. *J Ethnopharmacol* 79:165–168
19. Shukla YN, Anil S, Sunil K, Susil K (1999) Phytotoxic and antimicrobial constituents of *Argyrea speciosa* and *Oenothera biennis*. *J Ethnopharmacol* 67:241–245